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14. ABSTRACT

Our goal is to eliminate the tumor by vaccination and local ablation to render long-term immune protection without excessive autoimmune sequelae. Complimenting this regimen is systemic modulation of natural/induced Treg (iTreg) and intratumoral expression of immune augumenting molecules. The two aims are to
(1) Test the hypothesis that cryosurgery of cytokine enriched tumors amplifies Her-2 vaccine response, and
(2) Test the hypothesis that disabling iTreg conversion enhances Her-2 immunity, not autoimmunity.

In aim 1, we show that tumor destruction by cryoablation enables antigen presentation or manifestation of existing immunity. Treatment with CpG ODN immediately after cryoablation reduces local recurrence and improves systemic anti-tumor immunity. While pursuing Aim 2, we discovered that in addition to the loss of inducible Treg, TIEG1 deficiency also leads to increased mammary tumorigenesis in NeuT mice, indicating tumor suppressive activity.

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Table of Contents

Introduction

Our goal is to eliminate the tumor by combining vaccination with local ablation to render long-term immune protection without excessive autoimmune sequelae. Complimenting this regimen is systemic modulation of regulatory T cells (Treg) and intratumoral expression of immune augumenting molecules. We tested two related hypotheses in the specific aims.

Aim 1 Test the hypothesis that cryosurgery of cytokine enriched tumors amplifies Her-2 vaccine response.
Aim 2 Test the hypothesis that disabling iTreg conversion enhances Her-2 immunity, not autoimmunity

Body

Aim 1 Test the hypothesis that cryosurgery of cytokine enriched tumors amplifies Her-2 vaccine response. A manuscript describing the findings from this aim has been submitted for publication and is under revision.

To enrich the tumor microenvironment with immune stimulating cytokines, a toll-like receptor 9 (TLR9) agonist CpG oligodeoxynucleotide was injected peritumorally immediately following cryosurgery. A transient elevation of inflammatory cytokines was detected in the plasma of mice receiving CpG (Figure 7B of the attached manuscript). In BALB/c mice inoculated with TUBO (Figure 2E) or D2F2/E2 (Figure 5F) mammary tumor, local tumor recurrence following cryosurgery was reduced from 26% and 29% to 0% when CpG was incorporated in the regimen. Similarly, in Her2/neu-tolerant NeuT mice that bear TUBO tumor, addition of CpG to cryoablation reduced tumor recurrence from 71% to 12% (Figure 3C). Therefore, local immune stimulation reduced or eliminated residual tumor tissue after cryosurgery. Adaptive immunity is also elevated by CpG as demonstrated by heightened a-Her2/neu antibody levels in mice receiving both cryosurgery and CpG (Figure 3A).

Below is the manuscript submitted for publication

Cryotherapy with concurrent CpG Oligonucleotide treatment modulates Her2/neu immunity and controls local tumor recurrence

Veenstra JJ, Gibson HM, Littrup PJ, Reyes JD, Cher ML, Takashima A, and Wei WZ

Abstract

Percutaneous cryoablation is a minimally invasive procedure for tumor destruction, which can potentially initiate or amplify antitumor immunity through the release of tumor-associated antigens. However, clinically efficacious immunity is lacking and regional recurrences are a limiting factor relative to surgical excision. We evaluated α-Her2/neu immunity following cryoablation in BALB/c and neu-tolerant NeuT mice inoculated with neu or Her2 expressing mammary tumors TUBO and D2F2/E2 respectively. To promote immune activity local TLR9 activation via peritumoral CpG oligodeoxynucleotide injection was used with cryoablation. Cryoablation of TUBO primed the host to produce α-neu IgG rendering systemic protection,

which increased with the addition of CpG and resulted in a Th1-shifted response. Cryoablation of D2F2/E2 enabled systemic protection from tumor-induced immunity, but paradoxically promoted tumor growth relative to surgical excision if secondary tumor inoculation occurred during the tumor resolution phase. Addition of CpG to cryoablation partially abrogated effect. Cryoablation of TUBO in neu-tolerant NeuT mice did not significantly alter vaccine induced α-neu IgG or tumor protection. However, addition of CpG to cryoablation significantly reduced primary tumor recurrences from 71% to 12%. Similarly, primary tumor recurrences in BALB/c mice with TUBO and D2F2/E2 were reduced from 26% and 29% respectively to 0%. Treatment with CpG resulted in increased plasma inflammatory cytokines along with circulating dendritic and NK cells, reflecting innate immune activation. Therefore, cryoablation results in a range of antitumor responses dependent on tumor immunogenicity and host tolerance and benefits from peritumoral CpG treatment to enhance antitumor immunity and eradicate residual cancer at the treatment site.

INTRODUCTION

As immunotherapy becomes a mainstay in cancer therapy, attention is directed to immune constituents in the tumor microenvironment, particularly the modulation of their activities to enhance treatment outcome. In parallel with this progress is the advancement in image guided percutaneous cryoablation that utilizes ultra-cold temperatures to precisely destroy cancers of the breast, prostate, kidney, liver, bone, lung, brain, and skin (1). Cryoablation directly induces necrosis by damaging cell membranes and organelles via the formation of ice crystals, and indirectly through osmotic stress and ischemia from thrombosis of the microvasculature (2). Compared to surgical resection, cryoablation is minimally invasive, places less stress on the body, allows for quicker recovery, and is less costly (3). In addition to debulking the tumor, the necrotic tissue becomes a rich reservoir of tumor-associated antigens that are cleared by antigen presenting cells (APCs), creating a unique opportunity to prime or boost systemic antitumor immune responses, which may afford increased survival (4).

Induction of systemic immunity was initially observed in the 1970's when several patients had metastatic lesions regress following cryoablation of primary prostate tumors (5, 6). Further support of 'cryoimmunology' was linked to an increase in antibodies against DNA, RNA, and tumor cells in patients receiving palliative cryoablation for advanced cancer (7). More recently a study following 20 prostate cancer patients observed elevated levels of circulating inflammatory cytokines and cellular immunity after cryotherapy but found the response was transient and unable to prevent disease relapse (8). In a separate study, cryoablation of metastatic renal cell carcinoma resulted in elevated T-cell and antibody (Ab) responses without affecting the growth of untreated foci (9). While these results stimulated interest in the immunostimulatory potential of cryoablation, mechanisms leading to beneficial immunity have yet to be elucidated.

Although enhanced immune priming after cryoablation has been described in a number of pre-clinical studies (10-12), just as many studies indicate that cryoablation does not elicit any change in tumor-specific immunity (13-15), or worse, induces immune suppression and tumor progression (16-18). The inconsistencies in tumor-specific immunity and rejection of distant tumors reflect an inadequate understanding of the mechanisms of immune priming and suppression associated with cryoablation. The discrepancy in findings is, at least in part, due to the wide range of tumor models assessed and their varying immunogenicity in respective hosts.

To begin elucidating and exploiting the immunological mechanisms of cryotherapy, we evaluated antitumor immunity following cryoablation of BALB/c mouse mammary adenocarcinomas TUBO and D2F2/E2, which respectively express rat neu and human Her2 and exhibit well-characterized immunogenicity in wild type (WT) and neu tolerant transgenic mice. To further amplify and modulate cryoablation induced immunity, we also tested a Toll-like receptor (TLR) 9 agonist, CpG oligodeoxynucleotides. Dendritic cells (DC) and B-cells are the primary cell types that express TLR9, although mice have additional expression on monocytes and macrophages (19). Activation of these cells by CpG initiates stimulatory pathways that results in the indirect maturation, differentiation, and expansion of additional DCs, T-cells, NK cells, and macrophages (20-22). These cells subsequently secrete cytokines that generate a

pro-inflammatory and strongly Th1 biased environment (22, 23). These conditions enhance cytotoxic T-cell responses and inhibit Th2-mediated suppression, which is associated with the most efficacious antitumor immunity (24). Previous work by den Brok *et al* initially found that the addition of CpG to cryoablation results in more robust systemic tumor protection via increased DC maturation and cross-presentation of the model antigen OVA (25, 26). The study focused on DC activation but did not assess antigen specific Ab responses or other innate immune activation after treatment. In this study we provide a comprehensive evaluation of Her2/neu humoral and cell-mediated responses following cryoablation with or without CpG as well as tumor excision using multiple tumor systems in WT and immune tolerant mice. Importantly, we assessed the impact of peritumoral CpG injection on local recurrence which is a potential clinical limitation for cryoablation of locally aggressive or high-risk tumors (27, 28).

We show that cryoablation of neu$^+$ TUBO tumor in BALB/c mice resulted in immune priming, but had very little impact in neu tolerant NeuT mice. Cryoablation of Her2$^+$ D2F2/E2 enabled the functionality of tumor induced immunity but paradoxically promoted tumor growth if rechallenge occurred during the resolution phase of the cryoablated tumor. Peritumoral CpG injection immediately following cryoablation significantly elevated tumor specific Ab production and regional elevation in inflammatory cytokines in WT mice, and eliminated tumor recurrences in all scenarios. These findings suggest that cryoablation can trigger varying levels of antitumor responses depending on tumor immunogenicity and host tolerance, and that peritumoral CpG treatment enhances antitumor immunity and helps to eradicate residual local lesions.

MATERIALS AND METHODS

Mice: Female BALB/c mice (6-8 week old) were purchased from Charles River Laboratory. Heterozygous C57BL/6 pIL-1β-DsRed transgenic mice have previously been described (29). Heterozygous IL-1β-DsRed (BALB/c x C57BL/6) F1 mice were generated by crossing heterozygous C57BL/6 DsRed males with wild type (WT) BALB/c mice and screened for transgene expression by PCR. Heterozygous BALB/NeuT female mice, which express a transforming rat *neu* (30, 31) develop atypical ductal hyperplasia at 3 weeks of age which progresses to carcinoma *in situ* and then palpable tumors between 16 and 18 weeks of age (30, 31). Male NeuT mice do not develop mammary tumors. All animal procedures were approved by and performed in accordance with the regulation of Wayne State University Animal Investigation Committee.

Cell lines: The neu expressing TUBO mammary adenocarcinoma line, cloned from a spontaneous tumor in a NeuT female, grows progressively in normal BALB/c mice with similar histology as spontaneous NeuT tumors (32). The BALB/c D2F2 line was derived from a prolactin induced spontaneous mammary tumor (33). D2F2 cells transfected with WT human Her2 (34) were passaged in BALB/c mice to select D2F2/E2 that maintains Her2 expression *in vivo*. Antigen presenting cells (APC) 3T3/EKB and 3T3/NKB generated by transfection with K^d, B7.1 (CD80), and Her-2 (EKB) or neu (NKB) have been described (35).

Tumor Inoculation and DNA vaccination: Mice were inoculated with 2.5×10^5 tumor cells in mammary fat pad #4 (left) or #9 (right). Tumor growth was monitored by palpation and caliper measurement. Tumor volume was calculated as $v = (l \times w^2) / 2$. If tumors ulcerated or tumor burden became excessive mice were euthanized.

An admix of 30 μg each of pGM-CSF and pNeu-E2$_{TM}$ encoding a rat neu and human Her2 fusion protein or pVax1 (control) in 50 μL PBS was injected i.m. in the left gastrocnemius followed immediately by application of electrode gel and square wave electroporation using a BTX830 (BTX Harvard Apparatus) over the injection site (34).

Cryoablation and surgical procedures: Cryoablation was performed on tumors ~4x7 mm (~60 mm^3) in size, using the argon-based CryoCare system with the 1.7mm diameter PERC-15 Percryo CryoProbe – round ice (Endocare). Briefly, an ellipse of skin over the tumor was removed, and the tumor was retracted from the hind limb, peritoneal membrane, and adjacent skin without interrupting tumor vasculature. The cryoprobe was longitudinally inserted through the center of the tumor and freezing was initiated at 100% power. Each freeze cycle was administered for a full minute, reaching -150°C in the probe, followed by the thawing cycle, which lasted until the probe could be freed from the tumor (~1 minute). After two freeze-thaw cycles were completed, the skin was closed over the tumor. Sham surgery was performed without freezing and thawing. Surgical excision was performed using electrocautery to remove the tumor and adjacent mammary tissue. All incisions were closed using surgical staples.

CpG ODN mu2395: The murine specific class C CpG sequence: 5'-TCGACGTTTTCGGCGCGCGCCG-3' with a phosphorothioated backbone (Integrated DNA Technologies) was designed by substituting the human hexamer motif (5'-GTCGTT-3') for the optimal mouse motif (5'-GACGTT-3') in the C-Class ODN 2395 (36). One hundred μg of CpG was administered peritumorally over 3 injection sites: medial surface of adjacent musculature, caudual and rostral mammary tissue relative to tumor (10 μL/injection site).

Imaging and histology of cryoablated tumors: Tumors were removed from WT or IL-1β-DsRed BALB/c x C57BL/6 F1 mice and imaged immediately in the In-Vivo MS FX PRO (Carestream) in the Microscopy, Imaging and Cytometry Resources (MICR) core of Cancer

Center. Fluorescent spectra collected for 30 seconds were merged with white light images. Hematoxylin and eosin staining was prepared in the Animal Model and Therapeutics Evaluation Core. Images were recorded using the SCN400 slide scanner and software (Leica Microsystems).

Antibody measurement: Her2 and neu specific IgG levels in the serum were quantified by flow cytometry with a BD FACSCanto II (Becton Dickinson) (MICR core), using Her2 expressing SKOV3 cells or neu transfected 3T3/NKB cells as previously described (37). Normal mouse serum was a negative control. An Ab5 (a-Her-2 mAb TA-1, Calbiochem) or Ab4 (a-neu mAb, 7.16.4, Calbiochem) equivalent for Her2 and neu binding Ab, respectively, was calculated by regression analysis. Area under the curve (AUC) for Ab levels was measured for each mouse using the equation ((Day Y) – (Day X)) x (Ab X + Ab Y) / 2 between two time points where Day Y follows Day X. The sum of the values for all time points makes up the AUC.

***In vitro* antigen stimulation and multiplexing:** Lymph node cells (LNC) or splenocytes were enumerated using the Cellometer Vision (Nexcelom) and added to a 24 well plate ($8x10^5$ cells/well). 3T3/EKB or 3T3/KB were treated with 10 µg/mL Mitomycin C for 3 hours before co-incubation with LNC or splenocytes ($8x10^4$ cells/well). Supernatants were collected after 48 hours.

The levels of GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40/p70), and TNF-α were quantified in cell culture supernatant or plasma samples using the Cytokine Mouse Magnetic 10-Plex kit (Life Technologies) with the Magpix platform (Luminex) according to the manufacturer's instructions.

IFN γ ELISPOT: Antigen specific IFNγ production was measured by ELISPOT assay as previously described (37). Engineered APCs were incubated with LNC or splenocytes for 48

hrs. Spots were enumerated with the ImmunoSpot analyzer (CTL). Results were expressed as number of cytokine producing cells per 10^6 cells.

Cell phenotyping: PBMC phenotyping was performed with flow cytometry using a BD FACSCanto II (Becton Dickinson) (MICR core). Approximately $2x10^6$ PBMC were incubated for 15 min on ice in flow buffer (0.25% FBS in 1x PBS) with anti-mouse CD16/CD32 (2.4G2) (BD Pharmingen). Cells were subsequently stained with the eFluor 780 viability dye (eBioscience) and the following: α-CD4 (GK1.5), α-CD8α (53-6.7), α-TCRβ (H57-597), α-CD11c (N418), α-CD49b (DX5). All antibodies were from eBioscience unless otherwise indicated. Data were analyzed using FlowJo software (Tree Star). All populations enumerated as percentage of viable singlets.

Statistical analysis: Statistical analyses were conducted using GraphPad Prism 6. Error bars shown represent SEM unless otherwise noted. Survival percentages were calculated using the Kaplan-Meier method (38). *P* values less than 0.05, 0.01, and 0.001 are noted as *, **, and ***, respectively.

RESULTS

Necrosis and inflammatory infiltration following tumor cryoablation.

To evaluate cellular responses to cryoablation, BALB/c mice inoculated with neu$^+$ TUBO adenocarcinoma were treated with cryoablation and tumors were removed 1, 3, 9, and 29 days later for H&E histology (Fig. 1). Complete coagulative necrosis in the ablated tissue was evident by the absence of nuclear staining. Consistent with the classical wound healing process, polymorphonucleocytes (PMNs) in the peripheral and perivascular regions of the tumor were apparent 1 day after cryoablation (Fig. 1A) and dissipated by day 3 (39). Macrophage and fibroblast infiltration was evident by day 9 (Fig. 1A). Over the next 4 weeks, fibroblasts continued to expand and produce collagen, indicative of tissue remodeling.

To detect functional inflammatory infiltrates in cryoablated tumors, D2F2/E2 mammary tumor was inoculated into pIL-1β-DsRed transgenic (BALB/cxC57Bl/6 F1) mice, which utilize IL-1β promoter to drive the fluorescent marker gene DsRed. Mice received cryoablation or sham surgery when tumors reached ~60mm^3. On day 15 post-treatment, tissues were removed for *ex vivo* imaging on a Carestream MS FX Pro *in vivo* imager (Fig. 1B). Mean densitometry of tumor slices showed significantly greater DsRed fluorescence in cryoablated tumors relative to sham treated tumors (Fig. 1C). This finding provides direct evidence of IL-1β activation, consistent with infiltration of functional macrophages in ablated tissues.

Cryoablation of TUBO mammary adenocarcinoma induces α-neu IgG and systemic tumor protection.

To test if cryoablation induces systemic tumor immunity, female BALB/c mice were inoculated with neu$^+$ TUBO cells. When tumors reached ~60mm^3 they were treated with cryoablation with or without peritumoral CpG injection, CpG alone, surgical excision, or left untreated (n=6-8) (Fig. 2A). All tumors treated with cryoablation ± peritumoral CpG or surgical excision completely regressed except two mice in the cryoablation group that developed

recurrences on day 41 and 57. CpG treatment alone did not cause regression but reduced tumor growth relative to untreated mice (Fig. 2B).

Once cryoablated tumors had fully resolved (~8 wks), tumor-free mice received a secondary TUBO inoculation on the contralateral side to simulate outgrowth of a distant tumor. An additional group of naïve mice also received TUBO inoculation as a control (n=7). Cryoablation of the primary tumor protected 7/11 (64%) mice from secondary inoculation, whereas addition of CpG to cryoablation protected 15/16 (94%) mice (Fig. 2C-D). Thus, cryoablation of TUBO induced systemic antitumor immunity that was significantly enhanced by concurrent TLR9 stimulation via CpG. In contrast, surgical excision eliminated the primary tumor without triggering immune priming and only 1/6 mice rejected the secondary inoculation. These results suggest cryoablation, but not surgical excision released tumor associated antigens to prime tumor specific adaptive immunity.

In a fraction of cryoablation treated mice it was noted that primary tumors recurred but not if CpG was concurrently used. To further investigate this finding, results were compiled from 4 independent experiments to include mice treated with either cryoablation alone (n=28) or cryoablation and CpG (n=15). All mice were treated when tumors were ~ 60mm^3 and monitored for tumor recurrence for at least 30-90 days (Fig. 2E). Cryoablation alone had a recurrence rate of ~26%, occuring between 34-60 days. When CpG was combined with cryoablation the recurrence rate fell to 0%. Surgical excision of similar size tumors, produced no detectable recurrences (n=24) (data not shown). Therefore, equivalent long-term recurrence rates as surgical resection can be achieved with cryoablation if CpG is used concurrently.

To determine the mechanism of tumor rejection by cryoablation α-neu humoral and cellular responses were measured. Inoculation and growth of TUBO in untreated mice did not induce α-neu IgG by day 35 when tumors measured ~350 mm^3 (Supplementary Fig. S1 A), suggesting a block or lack of antigen presentation by the tumor alone. Mice treated with cryoablation produced increased levels of α-neu IgG beginning 14 days post-operatively (16±7 μg/mL) and plateaued thereafter (Fig. 3A). Peritumoral injection of CpG without cryoablation

also induced α-neu IgG (22±7 μg/mL), although tumors failed to regress. When CpG was used in combination with cryoablation, α-neu IgG levels continually increased to 58±16 μg/mL at day 41, which plateaued thereafter and remained elevated to at least day 70, indicating synergy between cryoablation and CpG injection. Area under the curve (AUC) analysis found significant differences occurring between cryoablation + CpG and cryoablation groups, as well as between cryoablation and excision groups. Mice undergoing surgical excision produced very low levels of α-neu IgG (1.5±0.03 μg/mL) similar to untreated tumor bearing mice (Fig. 3A). ELISPOT analysis of mice treated with cryoablation or cryoablation + CpG showed negligible α-neu IFNγ producing T-cells (not shown). Therefore, cryoablation induced primarily a humoral response which was enhanced by CpG treatment. Because rejection of neu-dependent tumors such as TUBO is mediated primarily through anti-neu Ab as reported by us and others (32, 35, 40), induction of α-neu Ab by cryoablation may be the primary mechanism for rejecting the secondary tumor. Additionally, cryoablation + CpG induced both IgG1 and IgG2a, whereas cryoablation alone induced primarily IgG1 (Fig. 3B). These results indicate cryoablation triggers a Th2 biased response which can be shifted toward a Th1 response with addition of CpG. However, CpG treatment alone was unable to mediate tumor regression despite elevated Ab levels, which argues for tumor debulking.

Cryoablation and α-neu immunity in neu tolerant BALB/NeuT mice.

Cryoablation of neu$^+$ TUBO was further tested in BALB/NeuT mice, which express a transforming rat *neu* and exhibit immune tolerance to neu (30, 31). Initial studies found cryoablation, with or without peritumoral CpG injection, insufficient to induce α-neu Ab, consistent with their immune tolerance to neu (not shown). As in breast cancer patients, moderate α-neu immunity can be induced in NeuT mice by DNA vaccination (41, 42).Therefore, we tested if cryoablation impacts vaccine induced immunity in these mice. To establish α-neu immunity, 10 wk old NeuT males were inoculated with TUBO and subsequently electrovaccinated using a hybrid rat neu / human Her2 DNA construct (pNeuE2) at 2 and 4 days

after TUBO inoculation (Fig. 4A). When tumors were ~60mm^3 in size mice were treated with cryoablation ± CpG or excision (n=8). The majority of tumors treated with cryoablation alone recurred after initial regression, whereas only 1/8 mice treated with cryo and CpG developed a recurrence (Fig. 4B). Results from 2 independent experiments using NeuT males compared recurrence rates of cryoablation alone (n=14), cryoablation and CpG (n=8), and excision (n=7). All mice were treated when tumors were ~60mm^3 and monitored for tumor recurrence for at least 40 days (Fig. 4C). Cryoablation alone had a recurrence rate of ~71%, with recurrences detected between 30-43 days after cryoablation. However, when CpG was combined with cryoablation the recurrence rate fell to 12%. No recurrences were detected with excision.

To simulate undetected systemic disease, mice received a secondary TUBO inoculation on the contralateral side 15 days after cryoablation. Neither cryoablation, with or without CpG, nor excision protected mice from secondary tumor growth (Fig. 4D-E). Accordingly, no modulation of vaccine induced α-neu IgG was observed after cryoablation ± CpG relative to tumor excision (Fig. 4F). Thus cryoablation, even with the addition of CpG did not boost vaccine induced antitumor protection. However the addition of CpG to cryoablation reduced recurrences to a similar level as that of excision, indicating a benefit of CpG despite the lack of adaptive immune modulation.

Cryoablation of D2F2/E2 mammary adenocarcinoma

The impact of cryoablation was further tested in D2F2/E2 mammary adenocarcinoma which, unlike TUBO, induces significant levels of α-Her2 IgG1 and splenocyte IFNγ response without exogenous intervention (Supplementary Fig. S2). However, this endogenous α-Her2 immunity is insufficient to prevent progressive tumor growth. BALB/c mice were inoculated with D2F2/E2 cells and tumors were treated on day 13 when they were ~60 mm^3 with cryoablation ± peritumoral CpG, CpG injection alone, tumor excision, or sham surgery (n=6-7) (Fig. 5A). All tumors treated with cryoablation ± peritumoral CpG or surgical excision completely regressed by day 41 with the exception of one mouse in the cryoablation alone group that developed a

recurrence on day 29. CpG treatment alone did not significantly change tumor growth relative to untreated mice (Fig. 5B).

Once cryoablated tumors had fully resolved (~7 wks), tumor-free mice received a secondary D2F2/E2 inoculation on the contralateral side to simulate outgrowth of a distant tumor. An additional group of naïve mice also received D2F2/E2 inoculation as a control (n=8). Treatment of the primary D2F2/E2 tumor with excision or cryoablation ± peritumoral CpG resulted in similar protection from the secondary inoculation (~70-85%) (Fig. 5C), suggesting that α-Her2 immunity induced by D2F2/E2 tumor growth renders systemic protection once the primary tumor is ablated, regardless of the treatment modality. At study completion (day 59), mice treated with cryoablation ± CpG or tumor excision produced comparable levels of IFNγ after *in vitro* Her2 stimulation of secondary TDLN, which was significantly elevated relative to naïve mice, illustrating host reactivity to D2F2/E2 tumor growth (Fig. 5D). Similar to TUBO, AUC analysis of α-Her2 IgG1 found no significant difference between treatment groups, but IgG2a levels were significantly elevated when cryoablation and CpG were used concurrently, further supporting the notion of a Th1 shifted response. These results indicate that α-Her2 immunity is induced by D2F2/E2 tumor growth and elicits systemic protection as long as the primary tumor is eliminated.

Similar to TUBO, primary D2F2/E2 tumors recurred after cryoablation in a fraction of mice, and these results were compiled from 4 independent experiments to include mice treated with either cryoablation alone (n=24) or cryoablation and CpG (n=25). All mice were treated when tumors were ~60mm^3 and monitored for tumor recurrence for at least 30 days and as long as 60 days (Fig. 6E). Cryoablation alone had a recurrence rate of ~29%, with recurrences detected between 24-58 days. When combined with CpG the recurrence rate fell to 0%, which suggests that CpG elevates antitumor immunity or modifies the tumor microenvironment to prevent recurrences.

Transient tumor promotion after cryoablation

Although cryoablation of D2F2/E2 protected mice from second inoculation once the ablated tumor had completely resolved, the presence of resolving necrotic tumor along with wound healing and inflammation may affect the growth of a co-existing tumor. Thus, we tested the level of tumor protection and growth during the wound healing phase. D2F2/E2 tumor-bearing mice were treated as previously described and a second inoculation was administered on day 13 during the wound healing phase (Fig. 6A). With cryoablation, 1/9 (12%) mice were protected from the second inoculation (Fig. 6B), compared to protection of 4/5 (80%) mice when inoculated after tumor resolution (day 41) (Fig. 5C). Addition of CpG in combination with cryoablation resulted in improved protection with 8/16 (50%) mice rejecting the second inoculation, compared to 5/6 (80%) after tumor resolution. Surgical resection protected 3/7 mice versus 5/7 mice when challenged after tumor resolution. Secondary tumors that grew in cryoablation ± CpG treated mice grew at comparable rates to untreated mice, while tumors in the excision group grew significantly slower relative to cryoablation alone (Fig. 6C). All groups had significantly delayed secondary tumor growth relative to naïve mice, indicating partial tumor inhibition by endogenous α-Her2 immunity (Fig. 6C). As expected, all tumor-experienced mice produced elevated α-Her2 splenocyte IFNγ responses relative to naïve mice, however there was no significant difference detected between treatment groups. These results suggest resolving necrotic tumor left by cryoablation may transiently promote tumor growth relative to tumor excision, which can partially be abrogated with the addition of CpG.

Peritumoral CpG treatment activates innate immunity

Peritumoral CpG injection reduces cryoablation recurrences independent from adaptive immunity as observed with NeuT mice (Fig. 4). To evaluate what changes are occurring with CpG administration cytokine responses were examined in plasma on day 2 and 12 after treatment with magnetic-bead multiplexing (Fig. 7A). On day 2, plasma from mice treated with CpG, either alone or in combination with cryoablation, showed significant increases in IL-1β, IL-6, IL-12, IFNγ, and TNFα relative to all other groups, indicating acute inflammation (Fig. 7B). By

day 12 most cytokines levels had subsided, with the exception of IL-12 which remained significantly elevated with CpG use.

Local and systemic tumor specific immunity was assessed from TDLNs and splenocytes harvested on day 12 with *in vitro* Her2 stimulation. IFNγ production from TDLN with CpG use, with or without cryoablation, was significantly elevated, but no significant differences were found between treatment groups in splenocytes, suggesting peritumoral CpG effects are primarily regionalized to the injection site with some residual activation of PBMC and transfer to plasma (Fig. 7C). In line with this notion, more than a 3x increase in circulating DCs ($CD11c^+$) was observed 2 days after peritumoral CpG treatment with or without cryoablation (Fig. 7D). Natural killer (NK) cells ($CD49b^+$) were also found to significantly increase after treatment with cryoablation and CpG. Similar to plasma cytokine levels, this increase in both DCs and NK cells was transient and dissipated by day 12 (not shown). These findings indicate peritumoral CpG injection strongly activates innate immunity and enhances regional adaptive responses.

DISCUSSION

To better understand the principles of cryotherapy and identify areas for therapeutic intervention, immunity induced by cryoablation was interrogated in two BALB/c mammary adenocarcinoma models expressing neu and Her2, TUBO and D2F2/E2 respectively. TUBO is induced by a constitutively activate rat neu and is sensitive to α-neu Ab, but growth of TUBO without intervention did not induce α-neu immunity. Thus, TUBO may resemble Herceptin sensitive breast cancer or others cancers with viral etiology or gene mutations that result in expression of neo-antigens the immune system can recognize but does not without exogenous manipulation (43). D2F2/E2 expresses human Her2 by transfection and is resistant to α-Her2 Ab but sensitive to T-cells. Growth of D2F2/E2 induces both humoral and cellular immunity which are not sufficient to elicit tumor regression. Thus, D2F2/E2 may be representative of $Her2^+$ tumors which are immunogenic but refractory to Her2 targeted therapy (44, 45).

Cryoablation of TUBO in WT mice resulted in immune priming, α-neu IgG production, and systemic protection in ~65% mice. In contrast to previous reports (4) resulting immunity was not Th1 biased but favored a Th2 response as evident by a dominant IgG1 Ab response. To reverse Th2 biased immunity, the Th1 promoting TLR9 agonist CpG was tested in combination with cryoablation. Cryoablation of TUBO with concurrent CpG injection resulted in a dramatic increase in α-neu IgG and shifted the response toward Th1, as evident by increased IgG2a levels. A similar, albeit less dramatic enhancement in Ab was also observed with D2F2/E2. This response protected nearly 100% of TUBO-bearing mice from secondary inoculation. These findings are further corroborated by Nierkens and den Brok, who reported similar enhanced protection and Th1 induction with combined CpG treatment (11, 25). Because the prominent immunological outcome of cryoablation is the induction or enhancement of Ab responses, this treatment modality may be especially useful for tumors sensitive to Ab-mediated cytotoxicity such as $Her2^+$ breast cancer.

Importantly, tumor recurrences of both TUBO and D2F2/E2 significantly dropped to 0% in WT mice and to 12% in NeuT mice when CpG was concurrently used. Interestingly, this effect appears to be independent of adaptive immunity based on the lack of immune modulation seen in NeuT mice, as well as cases of mice without tumor recurrence developing secondary tumors upon rechallenge (Fig. 5C). Furthermore, increased levels of cytokines, including IFNγ and IL-12, correlated with increased levels of circulating DCs and NK cells 2 days after CpG treatment, suggesting activation of innate immunity. Therefore, CpG may act to eliminate tumor recurrences by elevating local innate immune activation as well as adaptive immunity (24, 43). For high-risk tumors and oligometastatic disease, local recurrence is a significant clinical consideration for cryoablation (46, 47). Thus, the added protection provided with CpG may extend cryoablation to benefit a larger patient population.

Cryoablation of D2F2/E2 protected ~80% of mice if secondary inoculation was performed after the ablated tumor had been cleared (~6 wks). However, secondary inoculation before the ablated tumor had cleared (~2 wks) resulted in accelerated tumor growth relative to

surgical excision, suggesting transient promotion of tumor growth until the inflamed necrotic tumor has resolved. With concurrent CpG injection this effect was partially abrogated and tumor protection improved. To begin ruling out the possibility of cryoablation induced immunosuppression we analyzed circulating populations of T-regulatory cells, myeloid derived suppressor cells, as well as plasma and TDLN levels of IL-10 and TGFβ without finding any differences relative to excision treated mice (not shown). However, these findings do not exclude the possibility of another source of immunosuppression.

Our results, along with CpG clinical trial findings (19, 48), highly support concurrent CpG treatment with cryoablation to improve local tumor control with the potential to induce or amplify systemic tumor-specific immunity. Although not tested in this study, the use of therapeutics directly targeting immunosuppressive cells, such as Tregs (12, 49, 50), has enhanced cryoablation immune responses, and may further improve responses to cryoablation and CpG treatment, especially in tolerant hosts. Other means of redirecting tissue inflammation toward a Th1 response following cryoablation may also improve outcomes with cryoablation. As new immunotherapeutic options emerge, it becomes increasingly important to understand the mechanisms by which cryoablation affects antitumor immunity so an appropriate combination of therapeutic interventions can be used to achieve better clinical outcomes.

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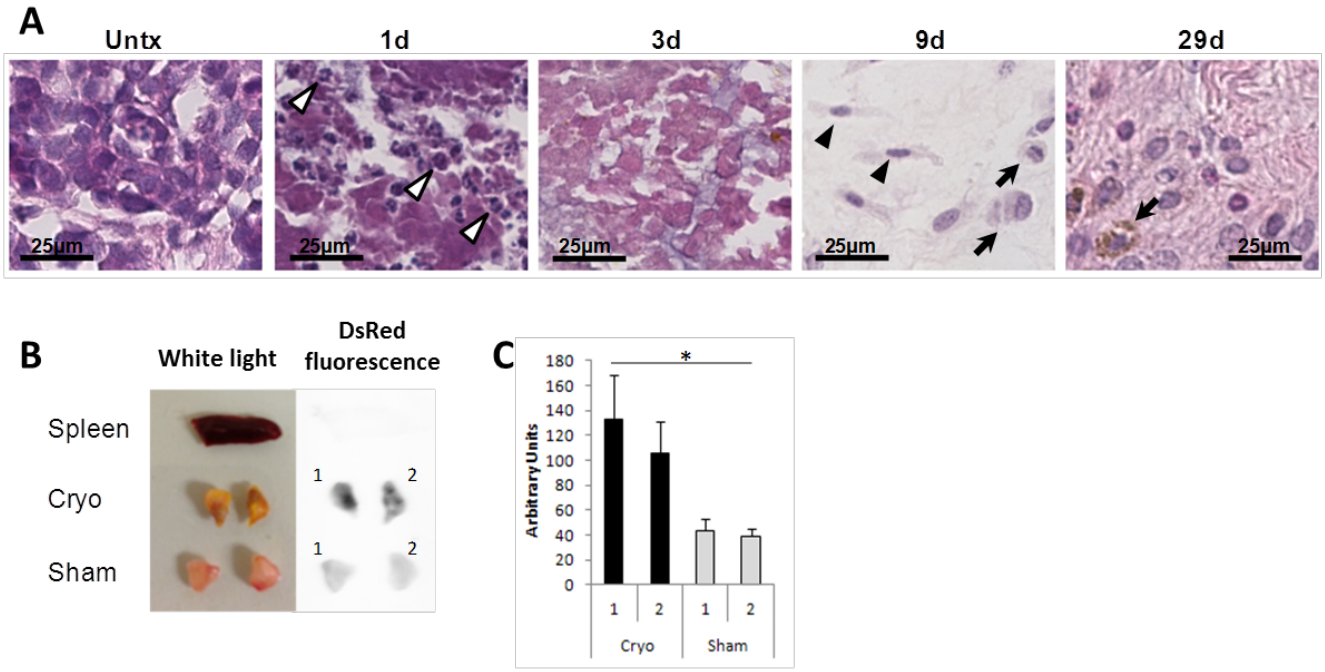


Figure 1. Necrosis and inflammatory infiltration following tumor cryoablation. (A) TUBO bearing BALB/c mice were treated with cryoablation with tumors harvested 1, 3, 9, and 29 days later for H&E histology (n=3-4). Untreated (Untx) tumor was collected for reference. Representative H&E 100x images shown. Polymorphonucleocytes (white arrow heads), fibroblasts (black arrow heads), and macrophages (arrows). (B) D2F2/E2 bearing BALB/c pIL-1β-DsRed transgenic mice were treated with cryoablation or sham surgery (n=2) and tissues were harvested 15 days later for *ex vivo* tumor imaging. Spleen was used as control. (C) Mean DsRed fluorescence of each tumor slice was quantified using ImageJ densitometry software. **P*<0.05 Unpaired t-test. Error bars, SD.

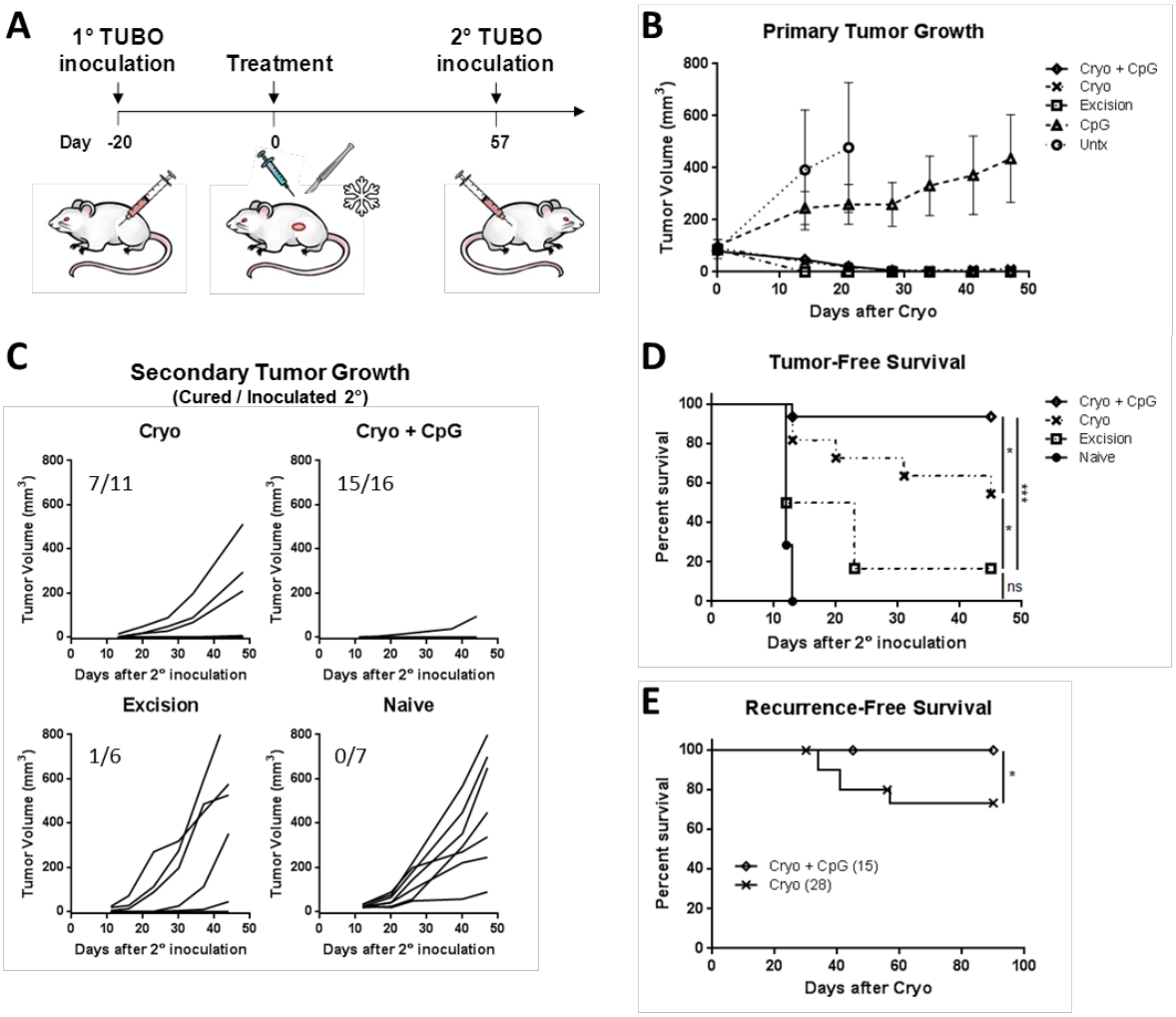


Figure 2. Cryoablation of an immunogenic tumor induces systemic antitumor protection and enhancement by CpG. (A) Experimental Scheme. (B) Primary tumor growth of TUBO. (C) Once cryoablated tumors had fully resolved (57 days), tumor-free mice received a second inoculation with TUBO on the contralateral side. Subsequent tumor growth was monitored, with any palpable tumor growth considered a failure in tumor protection. Number of mice (cured/inoculated with 2° tumor) shown. (D) Tumor-free survival after secondary TUBO inoculation. Data pooled from two independent experiments. Log-rank test. (E) Cryoablation recurrence data were pooled from 4 independent experiments, where all mice were monitored at least 30 days post-operatively for recurrence. Symbols indicate censored subjects within an experiment due to experimental endpoint. Long-rank test. **P*<0.05, ****P*<0.001. Error bars, SEM.

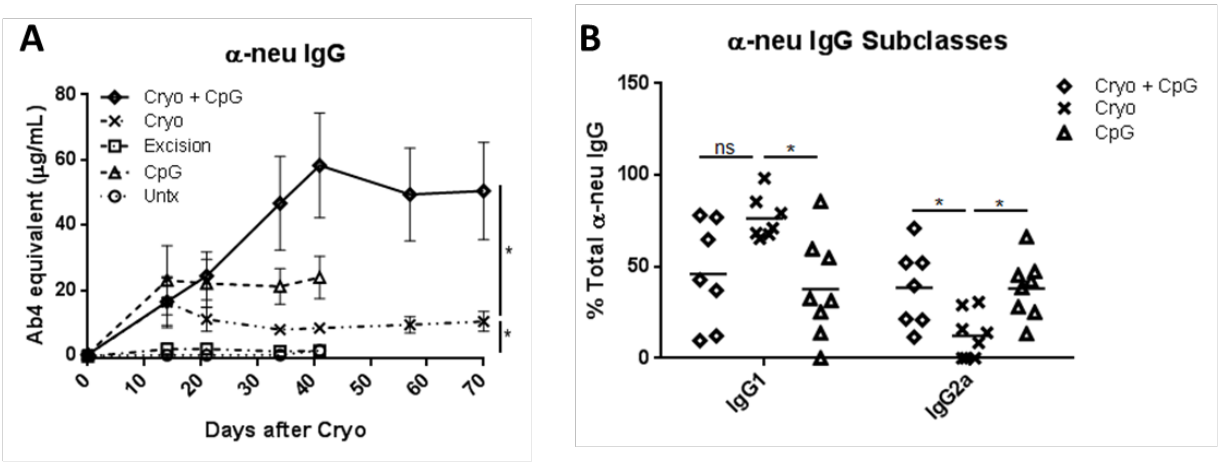


Figure 3. Cryoablation primarily induces α-neu IgG1 which is skewed toward IgG2a with the addition of CpG. Sera was collected throughout the experiment for α-neu IgG quantification. Area under the curve analysis was performed for (A) total α-neu IgG. Unpaired t-test. (B) Percent total α-neu IgG was calculated for IgG2a and IgG1 subclasses. Data shown (day 21) is representative of Ab subclass profile through day 70. One-way ANOVA with Tukey's post-test. **P*<0.05. Error bars, SEM.

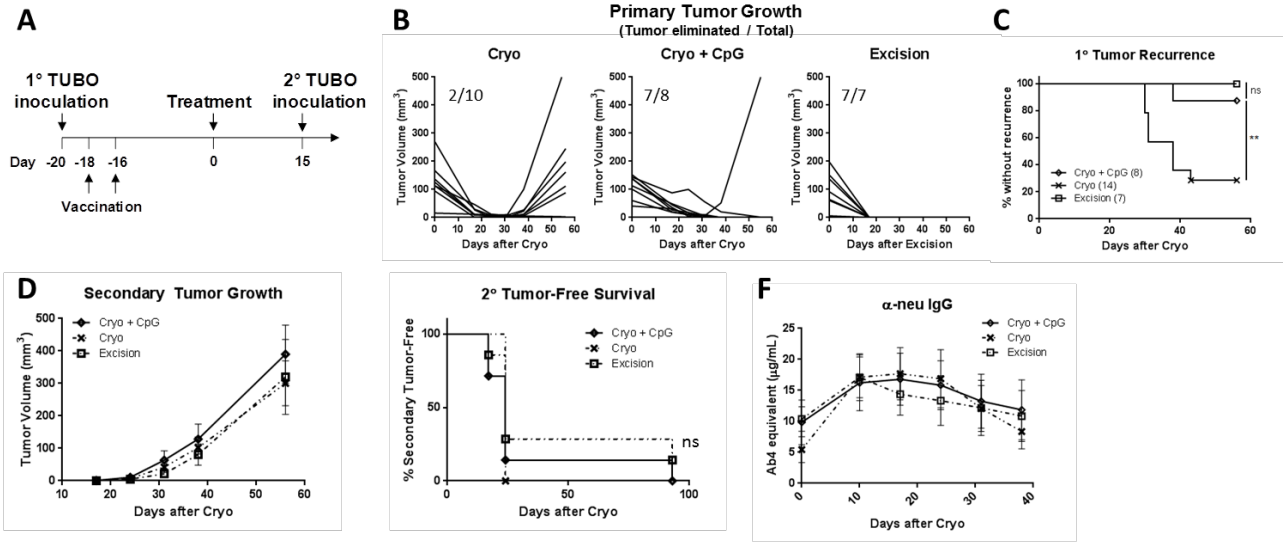


Figure 4. Cryoablation recurrences in tolerant NeuT mice are significantly reduced using CpG combination therapy. (A) Experimental scheme in male BALB/NeuT mice. All mice were vaccinated with an admix of 30µg each of pGM-CSF and pNeuE2 at day -18 and -16. Once tumors grew to ~60mm^3 (day 0) mice were treated with cryoablation, with or without peritumoral CpG, or tumor excision (n=8). Fifteen days after treatment, mice received a secondary TUBO inoculation on the contralateral side. (B) Primary tumor growth - mice (free of primary tumor/total) shown. (C) Any growth of primary tumor after treatment was considered a recurrence. Cryoablation recurrences were pooled from 2 independent experiments, where all mice were monitored at least 40 days post-operatively for recurrence. Symbols indicate censored subjects within an experiment due to experimental endpoint. Log-rank test. $^{**}P<0.01$. (D) Mean secondary tumor volume. (E) Protection from growth of secondary TUBO inoculation, where palpable secondary tumor growth was considered a failure in tumor-free survival. Log-rank test. Non-significant (ns). (F) Sera was collected for α-neu IgG quantification. Error bars, SEM.

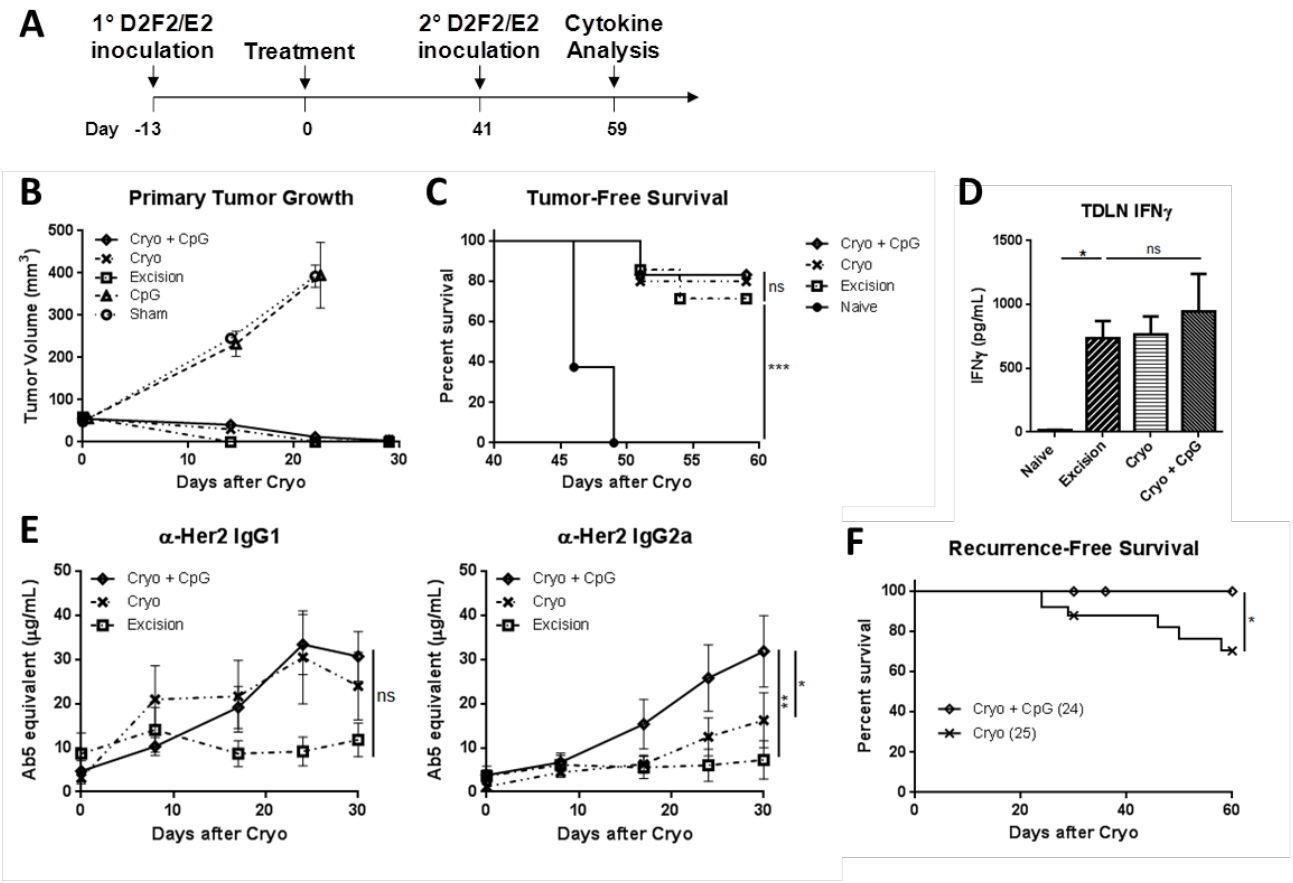


Figure 5. Complete resolution of D2F2/E2 tumor results in long-term protection. (A) Experimental scheme. (B) Mean primary tumor growth of D2F2/E2. (C) Once cryoablated tumors had fully resolved (41 days), tumor-free mice received a second inoculation with D2F2/E2 on the contralateral side. Subsequent tumor growth was monitored, with any palpable tumor growth considered a failure in tumor-free survival. Log-rank test. (D) Her2 stimulated TDLN supernatents were analyzed with Magpix based multiplexing for IFNγ. One-way ANOVA with Tukey's post-test. (E) Sera was collected throughout the experiment for α-Her2 IgG quantification. Area under the curve analysis was performed for α-neu IgG1 and IgG2a at day 30. One-way ANOVA with Tukey's post-test. (F) Cryoablation recurrence data were pooled from 4 independent experiments, where all mice were monitored at least 30 days post-operatively for recurrence. Symbols indicate censored subjects within an experiment due to experimental endpoint. Long-rank test. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars, SEM.

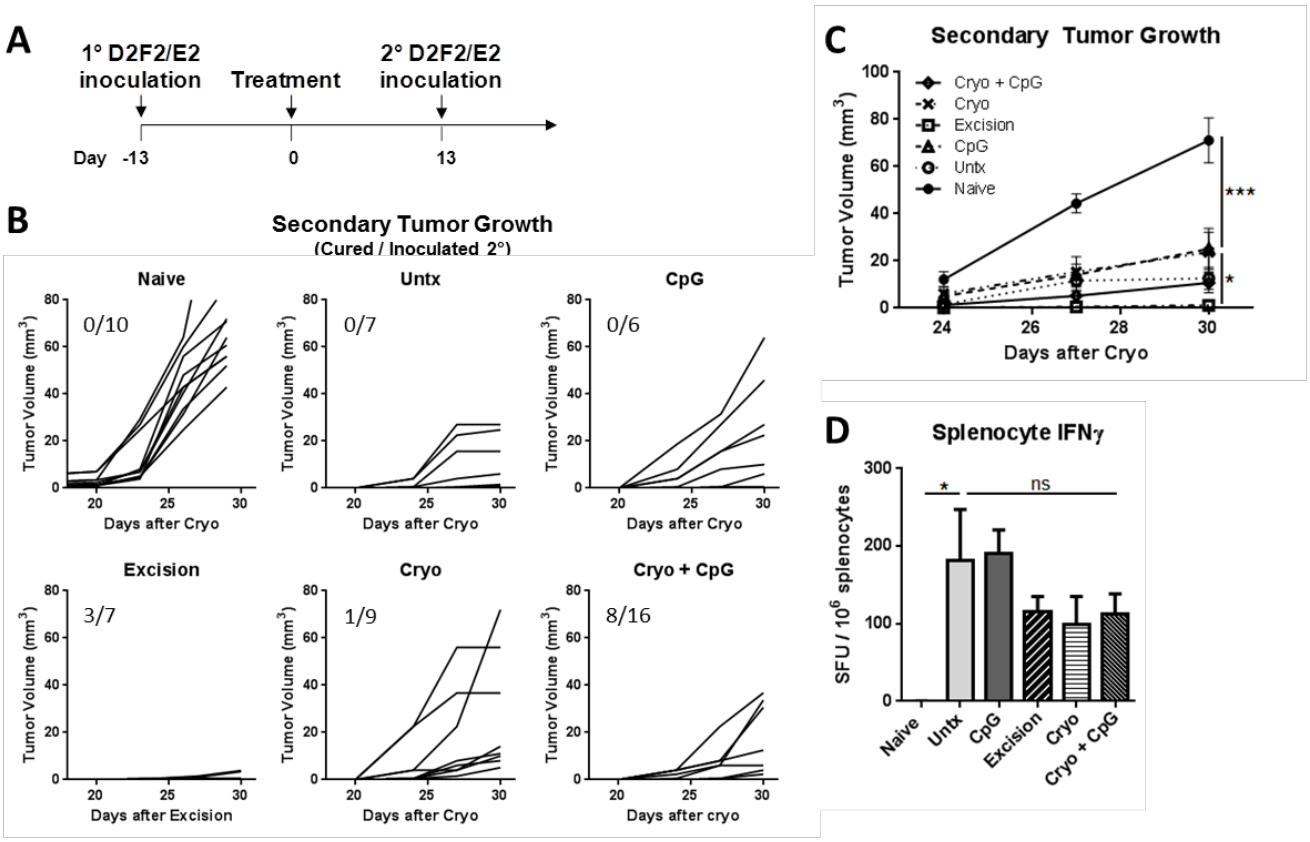
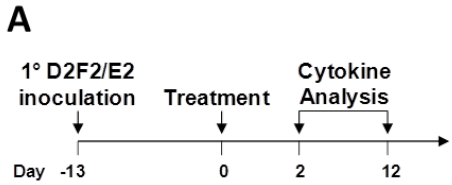


Figure 6. Cryoablation may result in transient immunosuppression, which can be overcome with CpG combination therapy. (A) Experimental scheme. (B) During the wound healing phase (13 days after treatment), mice received a secondary D2F2/E2 inoculation on the contralateral side. Subsequent tumor growth was monitored, with any palpable tumor growth considered a failure in tumor protection - mice with (eliminated secondary tumor/total) shown. (C) Mean secondary tumor volume. One-way ANOVA with Tukey's post-test. Data pooled from two independent experiments. (D) Spleens were harvested 30 days after cryoablation for IFNγ ELISPOT. Splenocytes were isolated and stimulated with either EKB or KB 3T3 lines for 48hr in an IFNγ ELISPOT. Results quantified in spot forming units (SFU) with KB subtracted from EKB. One-way ANOVA with Tukey's post-test. **P*<0.05, ****P*<0.001. Error bars, SEM.



B

Day 2 Plasma Cytokines (pg/mL)

Group (n)	IL-1β	IL-6	IL-12	IFNγ	TNFα
Naïve (5)	7±1	< 5	73±12	< 5	2±0
Sham (5)	2±0	7±7	49±7	12±9	2±0
CpG (6)	56±8*	26±5*	446±89*	488±151*	28±3*
Excision (6)	8±2	13±5	43±7	< 5	2±0
Cryo (6)	7±1	9±3	34±8	< 5	2±0
Cryo + CpG (7)	57±5*	32±5*	541±94*	501±86*	38±4*

Day 12

IL-12
73±12
57±6
188±24*
34±7
35±5
125±8*

* Significantly elevated above all groups without CpG treatment

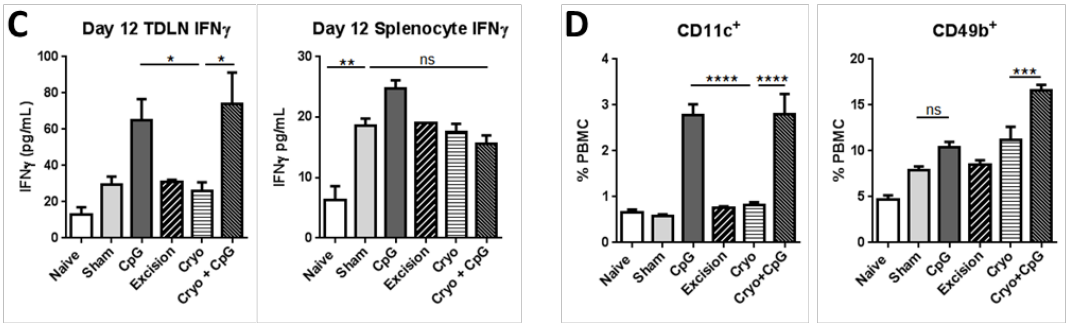


Figure 7. CpG treatment transiently increases inflammatory cytokines. (A) Experimental scheme. PBMC and plasma were collected 2 and 12 days after treatment with TDLN and splenocytes harvested on day 12 (from 2 independent experiments). PBMC, plasma, and LN from naïve mice were collected as a control. A total of $8x10^5$ LN cells were incubated with $8x10^4$ 3T3/EKB cells for 48 hours. (B) Plasma and (C) Her2 stimulated TDLN and splenocytes supernatents were analyzed with Magpix based multiplexing. One-way ANOVA with Tukey's post-test. (D) PBMCs were stained for TCRβ, CD4, CD8α, CD11c, and CD49b for flow cytometry analysis (day 2). $CD11c^+$ and $CD49b^+$ populations initially gated on $TCR\beta^-$ cells. One-way ANOVA with Tukey's test. **P*<0.05, ****P*<0.001 *****P*<0.0001. ± SEM.

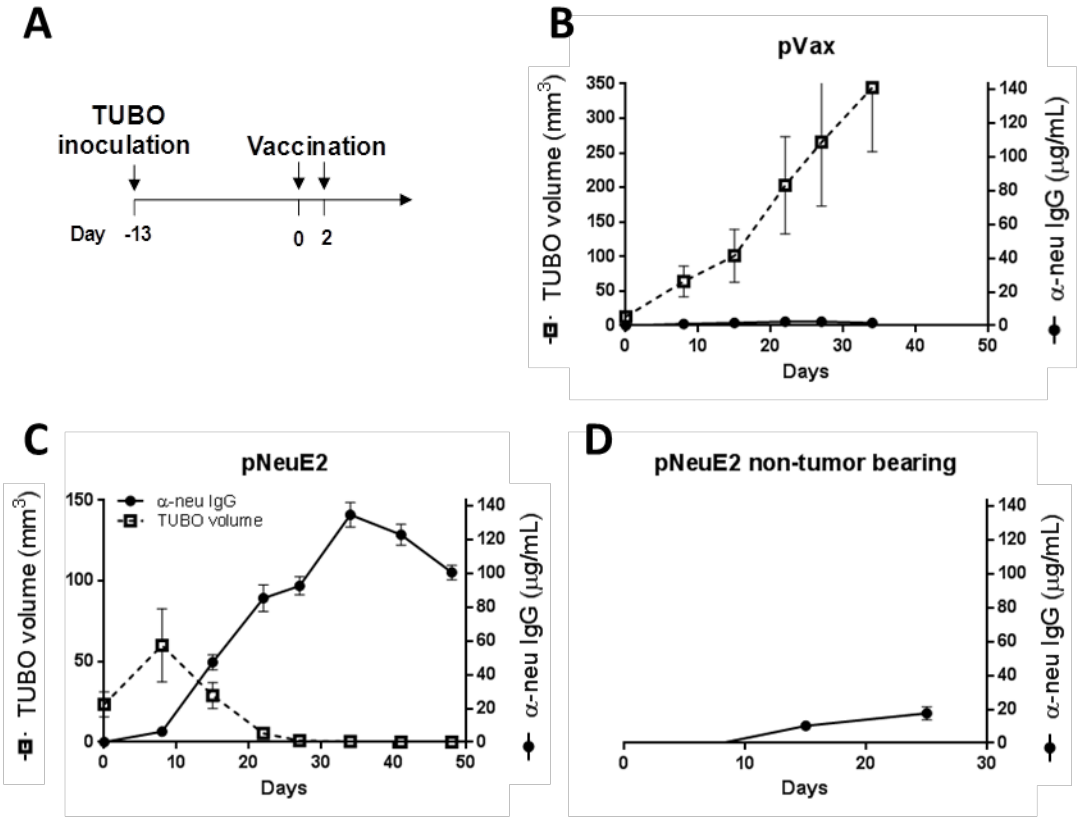


Figure S1. TUBO mammary adenocarcinoma is highly sensitive to α-neu Ab. (A) Experimental Scheme. BALB/c females were vaccinated with an admix of 30 μg of pGM-CSF and (B) 30 μg pNeuE2 or (C) 30 μg of pVax on day 0 and 2 (n= 4). Serum was collected for α-neu IgG quantification. TUBO volume (mm^3) and α-neu IgG (Ab4 equivalent μg/mL) are plotted on the left and right y-axis respectively. (D) Vaccination of non-tumor bearing BALB/c mice. Data representative of three independent experiments.

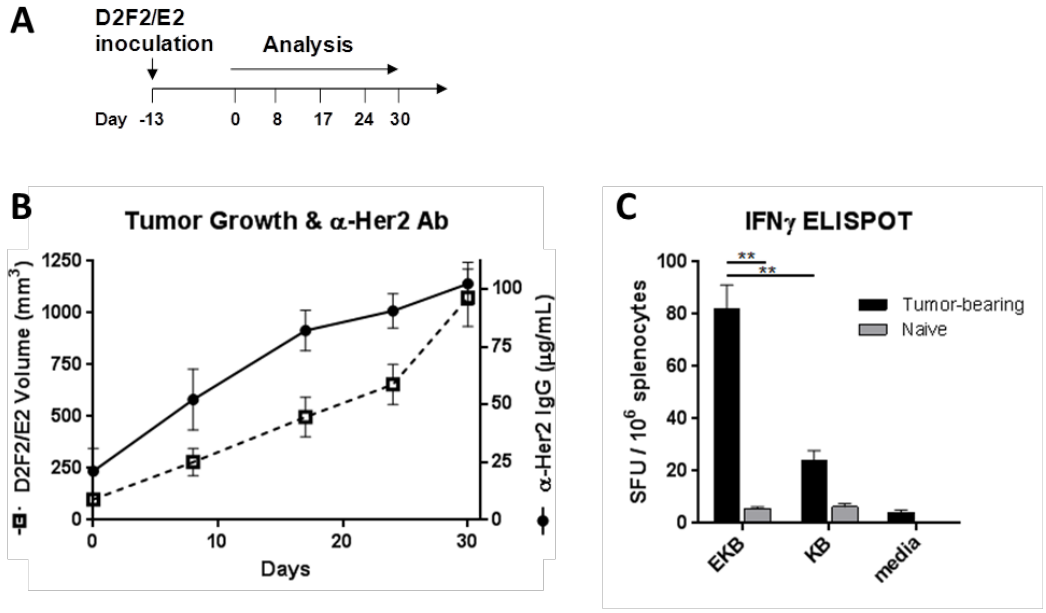


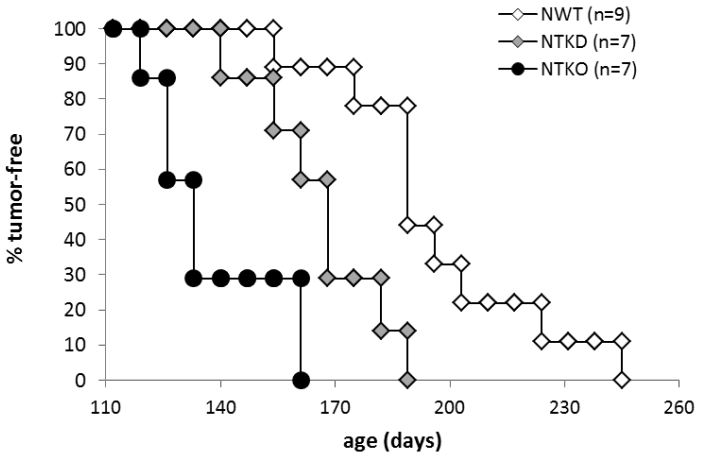
Figure S2. D2F2/E2 mammary adenocarcinoma induces α-Her2 immunity endogenously. (A) Experimental scheme. Sera and tumor measurements were collected from BALB/c mice on days 0, 8, 17, 24, and 30 (n= 9). (B) α-Her2 IgG levels were quantified from sera and correlated with tumor volume. Mean D2F2/E2 volume (mm^3) and α-Her2 IgG (Ab5 equivalent µg/mL) are plotted on the left and right y-axis respectively. (C) Three mice were euthanized at day 17 to collect spleens for IFNγ ELISPOT. Splenocytes were isolated and stimulated with either EKB or KB 3T3 lines for 48hr (n=3/group). Results quantified in spot forming units (SFU) per 10^6 splenocytes. $^{**}P<0.01$ Unpaired t-test. Error bars, SEM.

Aim 2 Test the hypothesis that disabling iTreg conversion enhances Her-2 immunity, not autoimmunity

TIEG1, also known as Kruppel-like transcription factor 10 (KLF10), is essential in TGF-β induced Treg development and bone morphogenesis. Previously, we showed reduced growth of TRAMP-C2 prostate cancer in TIEG1 k/o mice, suggesting enhanced α-tumor immunity with a reduction of TIEG1 mediated iTreg activity. To test if loss of TIEG1 impacts spontaneous tumorigenesis, C57BL/6 TIEG1 k/o mice were crossed with BALB NeuT mice that are heterozygous for a transforming rat neu and develop mammary tumors or salivary tumors. Contrary to the findings with transplanted TRAMP-C2 tumor, TIEG1+/-,NeuT F1 females develop mammary tumors 2-3 wks earlier than their littermates with intact TIEG1. When TIEG1+/-,NeuT F1 mice were back-crossed with TIEG1 k/o mice to produce TIEG1 k/o, NeuT mice, their mammary tumorigenesis was further accelerated.

This accelerated mammary tumorigenesis, with concurrent reduction of iTreg generation, suggests direct and strong inhibition of spontaneous tumorigenesis by TIEG1. Since TGF-β suppresses early phases of mammary tumor progression, TIEG1 may be a critical downstream factor in this pathway also. Analysis of tyrosine kinase activity by RTK signaling array showed similar activation of MAPK, AKT and S6 ribosomal protein in mammary tumors from TIEG1+/- or TIEG1+/+ NeuT mice, suggesting intact neu signaling whether TGF-β signaling is altered. To test the impact of vaccination on tumor progression, male TIEG+/- NeuT F1 and control TIEG1+/+ NeuT F1 mice were electrovaccinated with pNeuE2 which encodes a fusion protein of rat neu and human Her2, to generate comparable levels of α–neu Ab. But, only 3/7 NeuT males with intact TIEG1 developed mammary tumors after vaccination, while 9/10 TIEG1+/- NeuT males developed 1-2 mammary tumors. These results strongly indicate TIEG1 as a tumor suppressor in mammary tumorigenesis. These findings identify TIEG1 as a key factor in

multiple signaling pathways that profoundly impact tumor growth and immunity. In addition to regulatory iTreg generation, TIEG1 also renders tumor suppressive activity to warrant further investigation.



NeuT mice were crossed with TIEG1 knockout mice to produce three genotypes: NeuT/WT (NWT), NeuT/TIEG1$^{+/-}$ (NTKD), and NeuT/TIEG1$^{-/-}$ (NTKO). Tumor growth is monitored and measured every week. Tumor-free survival curve depicts TIEG1 deficiency accelerated spontaneous tumorigenesis in NeuT mice.

Key Research Accomplishments

1. Demonstrate reduced local recurrence and elevated systemic immunity by tumor cryoablation in combination with TLR9 agonist CpG.
2. Discover an acceleration rate of mammary tumorigenesis in mice with reduced TIEG1 expression, suggesting tumor suppressor activity of TIEG1.

Reportable Outcomes

*Venuprasad, P., and Wei, WZ. Modulation of HER-2 DNA vaccine response by tumor cryosurgery and abrogation of inducible regulatory T cells (iTreg) conversion. DOD Breast Cancer Program, Era of Hope meeting, 2011.

*Veenstra, J, Littrup, P, Wei, WZ. Amplification of tumor immunity with cryoablation. Proc. AACR, 2011.

*Kong, Y.M., Brown, N.K., Flynn, J.C., McCormick, D.J., Brusic, V., Morris, G.P. and David, C.S. Efficacy of HLA-DRB1*03:01 and H2E transgenic mouse strains to correlate pathogenic thyroglobulin epitopes for autoimmune thyroiditis. J. Autoimmunity. 37:43-70, 2011.

Conclusions

Tumor destruction by cryoablation enables antigen presentation or manifestation of existing immunity. Treatment with CpG ODN immediately after cryoablation reduces local recurrence and improves systemic anti-tumor immunity. This combination therapy can be translated to clinical practice to improve treatment outcome. In addition to the loss of inducible Treg, TIEG1 deficiency also leads to increased mammary tumorigenesis in NeuT mice, indicating tumor suppressor activity.